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OPIATE RECEPTOR BINDING PROPERTIES OF CARFENTANIL

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RESEARCH DIRECTORATE

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Efforts to increase the safety margin of opiate-type drugs led to the discovery of the fentanyl series of compounds. These compounds are sufficiently potent to produce surgical anesthesia with few of the negative side effects associated with morphine, except for respiratory depression. Previous research demonstrated a pharmacological dissociation between the analgesic and respiratory effects of narcotics that is based on different types of the opioid receptor. This study assessed the relative potency and selectivity of one fentanyl derivative (carfentanil) for the putative MU, KAPPA, and DELTA types of opioid receptor. Rat brain tissue homogenates were incubated with ^{3}H-Dihydromorphine, ^{3}H-D-Ala-D-Leu Enkephalin or ^{3}H-Ethylketocyclazocine to label the MU, DELTA, and KAPPA receptor sites, respectively. Carfentanil's apparent affinity for each receptor was estimated from the concentration required to displace 50% of the specifically bound radioligands. Biphasic displacement curves were observed for each radioligand, which suggest high and low affinity binding sites. (continued on reverse)

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19. Abstract (continued)

Carfentanil appeared equipotent in displacing the MU and KAPPA radioligands with IC_{50}s of 0.7 and 100 pM, while displacing the DELTA radioligand with IC_{50}s of 0.8 and 40 nM. The results are discussed in terms of their significance for explaining the persistence of respiratory depression of the pharmacologic profile of the fentanyl series of compounds.

PREFACE

The work described in this report was authorized under Project 21085000A173, Resolution of the Incapacitating and Respiratory Depressive Mechanisms of Fentanyl Derivatives: A Receptor Binding Study. This work was started in January 1985 and completed in June 1986. The experimental data are contained in laboratory notebooks 85-0005 and 85-0138.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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1. INTRODUCTION

The use of morphine at dose levels necessary to produce surgical anesthesia offers certain advantages over volatile anesthetics: greater stability in cardiovascular dynamics, mitigation of the surgical 'stress' response, and the desired postoperative analgesia.[1,2] The major drawback to narcotic anesthesia is the small margin between the dose of morphine required to induce anesthesia and the amount that causes death. Because of the benefits of narcotic anesthesia, considerable research was done to increase the margin of safety between a narcotic's therapeutic and lethal dose. Increments in the safety margin of opiate-like compounds are generally associated with an increase in drug potency: the more potent the compound, the lower the dose required to elicit the desired effect. This also means there is less drug to produce the undesirable 'nonspecific' side effects.

The most potent family of narcotics synthesized to date are the fentanyl derivatives of 4-anilinopiperidine (see Figure 1 and the table).[3] With analgesic potencies up to 8,000 times that of morphine, a peak effect within minutes, and a duration of effects that can range from minutes to hours depending on the particular compound;[3] the fentanyls have been used as both adjuvants and the sole anesthetic agent in certain types of surgery.[1,3] Like all other morphinomimetics, however, anesthetic doses of the fentanyls depress respiratory function and can be lethal in the absence of ventilatory assistance. Merely increasing drug potency does not appear to be a sufficient pharmacological variable for adequately dissociating between the anesthetic and respiratory effects of narcotics.

One of the central dogmas of molecular pharmacology is that the spectrum of a drug's action reflects its various affinities for, and access to, different biological receptors. Up to five topologically distinct types of opioid receptors are proposed in the literature;[4,5] the MU, DELTA, and KAPPA receptor types are the most extensively characterized. The physiologic and behavioral rationale and in vitro receptor binding profiles for the MU, DELTA, and KAPPA receptors are in several reviews.[4,6] The existence of multiple opioid receptor types theoretically allows greater specificity of drug effect because researchers can develop compounds that exhibit greater affinity for one receptor type over the others. Although the pharmacological literature shows that increasing drug potency can lessen the spectrum of a drug's physiological effects, it does not show to what extent drug potency translates into receptor selectivity in those cases where multiple types of the opioid receptor population are involved.

This study assessed the degree to which one of the most potent congeners of fentanyl (carfentanil) exhibits selectivity for the MU, DELTA, and KAPPA types of the opioid receptor. The procedure compared the ability of unlabeled carfentanil to displace the specific binding of several radioligands to rat brain membranes in vitro.

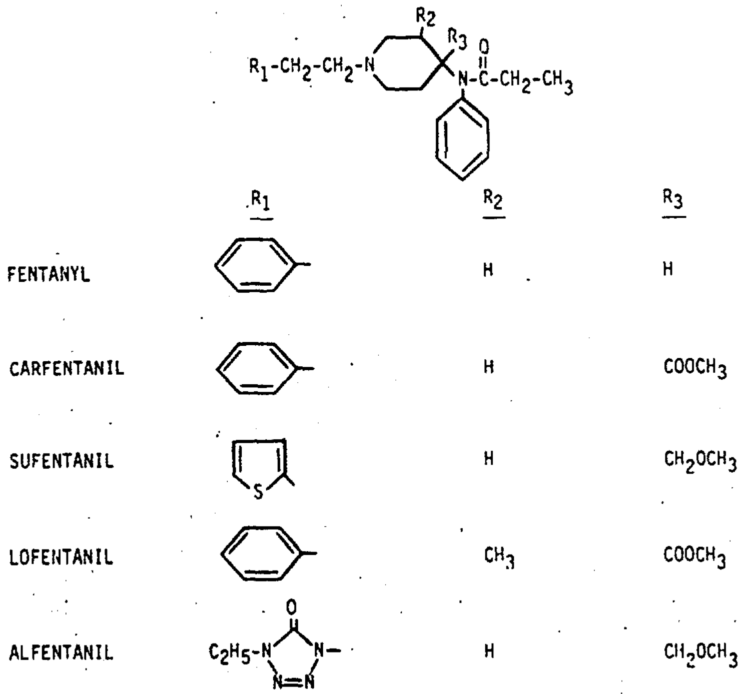

Figure 1. Chemical Structures of N-4-Substituted (1-2-Arylethyl)-4-Piperidinyl-N-Phenyl Propanamides[3]

Table. Some Effect and Safety Characteristics of the Fentanyls*

Compound	Lowest ED50 (mg/kg)	Potency Ratio	LD50 (mg/kg)	Safety Margin	Peak Effect (min)
Morphine	3.2	1	223	71	30
Fentanyl	0.01	320	3.1	277	4
Alfentanil	0.04	80	48	1080	1
Sufentanil	0.0007	4571	18	26716	8
Lofentanil	0.0006	5333	0.07	112	8
Carfentanil	0.0004	8000	3.1	8460	10

*Adapted from Janssen.[3] All values are for rats after intravenous injection. Lowest ED50 is based on tail-withdrawal test.

2. MATERIALS AND METHODS

2.1 Chemicals.

2.1.1 Carfentanil.

Carfentanil, methyl 4-((1-oxopropyl)phenylamine)-1-(2-phenethyl)-4-piperidinecarboxylate, was obtained from the Research Directorate, U.S. Army Chemical Research, Development and Engineering Center, as the oxalate salt wlth greater than 99% purity.

2.1.2 Bremazocine.

Bremazocine, (-)-5-ethyl-9,9-dimethyl-2-(1-hydroxy-cyclopropyl-methyl)-2'hydroxy-6,7 benzomorphan, was generously donated by D. Romer of Sandoz, Ltd.

2.1.3 Isotopes.

^{3}H-Dihydromorphine (83.3 Ci/mmol), ^{3}H-Ethylketocyclazocine (22.5 Ci/mmol), and ^{3}H-D-Ala-D-Leu Enkephalin (46.9 Ci/mmol) were purchased from New England Nuclear.

2.2 Animals.

Male albino rats (Fisher 344), weighing 200-300 g, were used as the source of brain tissue in the study. The rats were procured through the U.S. Army Medical Research Institute of Chemical Defense and were individually housed under a 12-hr light:dark cycle with lights on at 0700. The vivarium was maintained at 23 $\pm$ 3 °C and 65% relative humidity. Purina rat chow and water were available *ad libitum*.

2.3 Methods.

2.3.1 Tissue Preparation.

Preparation of brain tissue for receptor binding consisted of homogenizing a freshly dissected rat brain, minus cerebellum and brainstem, in 20 volumes 50 mM tris-HCl buffer (pH 7.7) with a Teflon and glass tissue homogenizer. The homogenate was subjected to a second homogenization with a Brinkman Polytron (setting 6, 10 sec) and centrifuged (45,000 x g) for 20 min at 4 °C. The resulting pellet was resuspended in 50 volumes of tris buffer using the Polytron (setting 6, 10 sec) and incubated with gentle agitation at 37 °C for 30 min. The suspension was centrifuged once more (45,000 x g) for 20 min, and the pellet was resuspended in 50 volumes of fresh buffer. Each incubation tube received 0.5 ml of the final tissue suspension, equivalent to 20 mg original wet tissue.

2.3.2 Receptor Binding Protocol.

All incubations of tissue with ^{3}H-ligand were performed in a final volume of 1 ml. Stock concentrations of ^{3}H-Dihydromorphine (^{3}H-DHM),

^3H-Ethylketocyclazocine (^3H-EKC), and ^3H-D-Ala-D-Leu Enkephalin (^3H-DADLE) were diluted 100-fold to give final concentrations of 1 nM. Specific binding of each radioligand to opioid receptor sites was defined by the amount displaced by 100 nM bremazocine. Incubations were initiated by adding 0.5 ml of the tissue suspension to 0.5 ml of buffer containing appropriate concentrations of radioligand, carfentanil, or bremazocine. The tubes were vortexed and set at room temperature (25 °C) for 60 min. The samples were aspirated onto Whatman GF/B filter strips using a Brandel Cell Harvester and washed three times with 5 ml cold tris buffer. Filter discs were placed in plastic scintillation vials containing 5 ml of Formula 947 (New England Nuclear). The vials were dark and cold adapted prior to counting in a Packard Tri-Carb Scintillation Spectrometer.

2.3.3 Data Analysis.

The results represent the mean of three independent experiments with each point run in duplicate during each experiment. For graphical representation, curves were fit to the data using a Hewlett Packard 41CV calculator with a statistics package (HP-41C STAT PAC). Displacement curves were best fit according to a power function ($R^2 > 0.9$). The Hill coefficients (i.e., slope of the Hill plots) were calculated by linear regression. The Hill binding constant (K'_D), an estimate of carfentanil's equilibrium binding constant, was calculated from the Hill plots as the abcissa value where $LOG_{10}(P/100-P) = 0$, and P = percent of specifically bound ^3H-EKC, ^3H-DHM, or ^3H-DADLE.

3. RESULTS

3.1 MU Binding.

Figure 2 shows the results of carfentanil's ability to displace ^3H-DHM from the putative MU class of opioid receptors. Membranes were incubated with 1 nM ^3H-DHM and 10^{-6} - 10^{-14} M carfentanil. Specific binding of ^3H-DHM was defined by 100 nM bremazocine and represented 56% of total ^3H-DHM bound. Carfentanil produces a biphasic inhibition of membrane bound ^3H-DHM, suggesting two classes of saturable binding sites. Carfentanil inhibits binding of ^3H-DHM with an IC_{50} of 0.0006 nM for the first class of sites and an IC_{50} of 0.087 nM for the second class of sites. A Hill plot of the data (Figure 3) yields a slope of 0.5 and K'_D value of 8.9 pM.

3.2 KAPPA Binding.

Figure 4 shows carfentanil's ability to displace ^3H-EKC from the putative KAPPA opioid receptor type. Membranes were incubated with 1 nM ^3H-EKC and 10^{-6} - 10^{-14} M carfentanil. Specific binding of ^3H-EKC was defined by 100 nM bremazocine and represented 90% of total ^3H-EKC bound. Similar to that observed with ^3H-DHM, carfentanil inhibits ^3H-EKC binding in a biphasic manner with an IC_{50} of 0.0008 nM for the first component of the curve and an IC_{50} of 0.125 nM for the second component. The slope of the Hill plot is 0.51 (Figure 5) with K'_D of 7.1 nM.

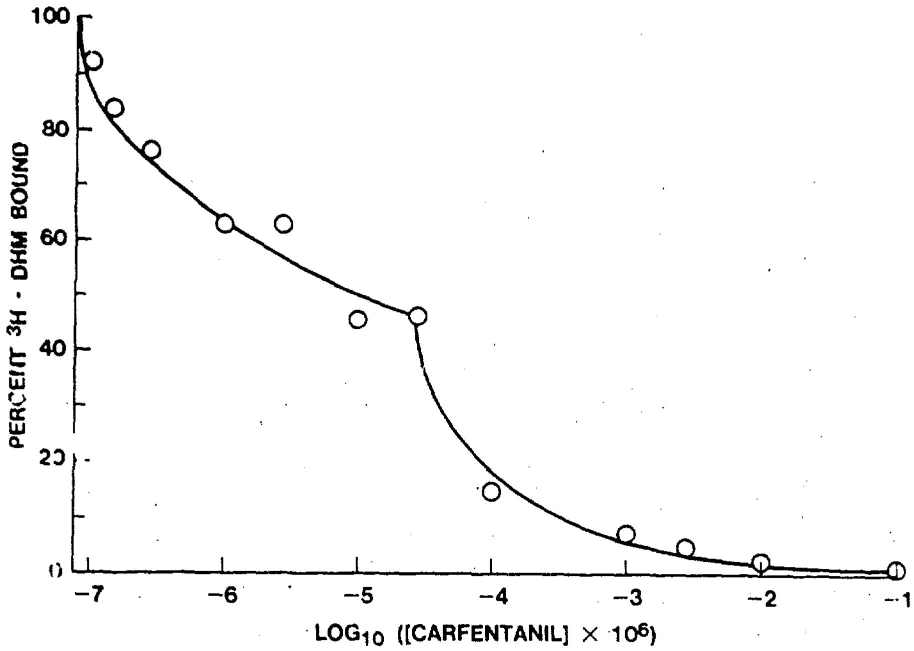

Figure 2. Displacement of ^{3}H-DHM Binding by Carfentanil

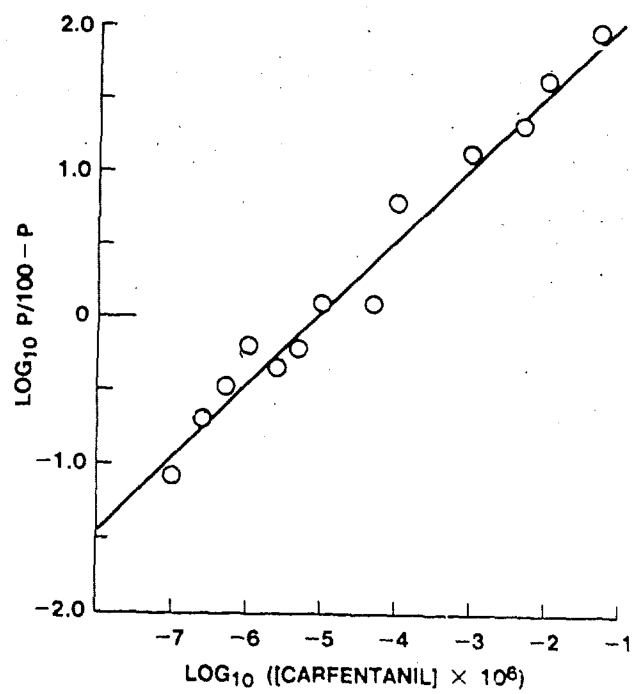

Figure 3. Hill Plot of ^{3}H-DHM Displacement by Carfentanil

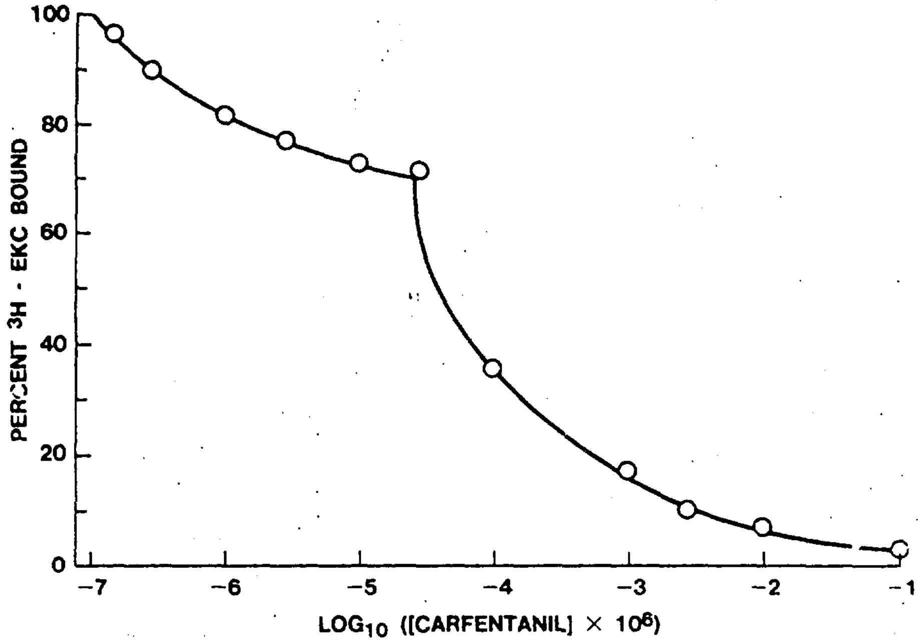

Figure 4. Displacement of ^{3}H-EKC Binding by Carfentanil

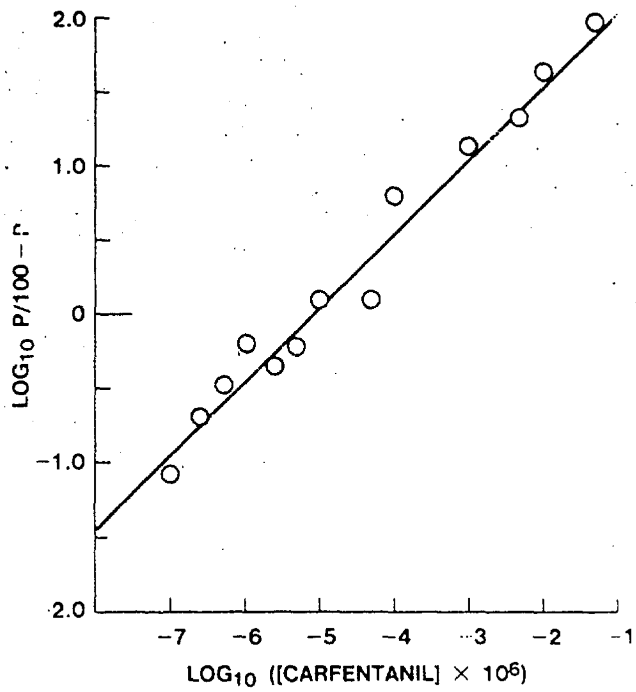

Figure 5. Hill Plot of ^{3}H-EKC Displacement by Carfentanil

3.3 DELTA Binding.

The displacement of membrane-bound ^{3}H-DADLE by carfentanil also displays a biphasic curve (Figure 6). Membranes were incubated with 1 nM ^{3}H-DADLE and 10^{-6} - 10^{-14} M carfentanil. Specific binding of ^{3}H-DADLE was defined by 100 nM bremazocine and represented 63% of total ^{3}H-DADLE bound. Carfentanil displaces the first component of ^{3}H-DADLE binding with an IC_{50} of 0.75 nM and displaces the second component with IC_{50} of 40 nM. The Hill plot (Figure 7) yields a slope of 0.58 with an apparent K'_D of 7.1 nM.

3.4 Selectivity Profile.

The relative selectivity of a compound for one receptor over another can be defined by the ratio of its inhibition constants (K_I) for displacing radioligands from the different receptors.[7] The inhibition constants for carfentanil were calculated by the method of Cheng and Prusoff[8]

$$K_I = IC_{50}/1+S/k_M$$

where

S = concentration of isotope used in the competition experiment

k_M = the dissociation constant of the isotope.

Previous analyses of saturation curves for each of the isotopes show high and low affinity binding sites with apparent K'_D values of 0.5 and 2 nM (^{3}H-DHM), 0.6 and 3 nM (^{3}H-EKC), and 1 and 5 nM (^{3}H-DADLE). For the high affinity component of the displacement curves, carfentanil exhibits a K_I of 0.3 pM for displacing ^{3}H-DHM and ^{3}H-EKC and a K_I of 0.5 nM for displacing ^{3}H-DADLE. For the low affinity components, carfentanil displaced ^{3}H-DHM, ^{3}H-EKC, and ^{3}-DADLE with inhibition constants of 0.06, 0.09, and 26.7 nM, respectively. By establishing the K_I of the highest affinity site (i.e., ^{3}H-DHM) as the denominator in the ratio to express receptor selectivity, the selectivity profile of carfentanil between the high affinity sites is 1:1:1666 (MU:KAPPA:DELTA) and 1:1.5:445 (MU:KAPPA:DELTA) for the low affinity sites.

4. DISCUSSION

This study assessed the potency and relative selectivity of carfentanil's interaction with the MU, KAPPA, and DELTA opioid receptors. For each radioligand used to define the receptors, carfentanil produced biphasic inhibition curves and Hill coefficients significantly less than 1. Both features of the data may be interpreted to indicate that each of the radioligands bind to more than one type of receptor. The ability of ^{3}H-DHM, ^{3}H-EKC, and ^{3}H-DADLE to label more than one class of receptor was noted previously.[9,10] This may preclude one from drawing definitive conclusions regarding carfentanil's selectivity for the different receptor types; however, given the current procedures, certain features of the data are of significance in attempting to understand how carfentanil and other fentanyl derivatives differ from less potent opiate-type compounds in their association with opioid receptors.

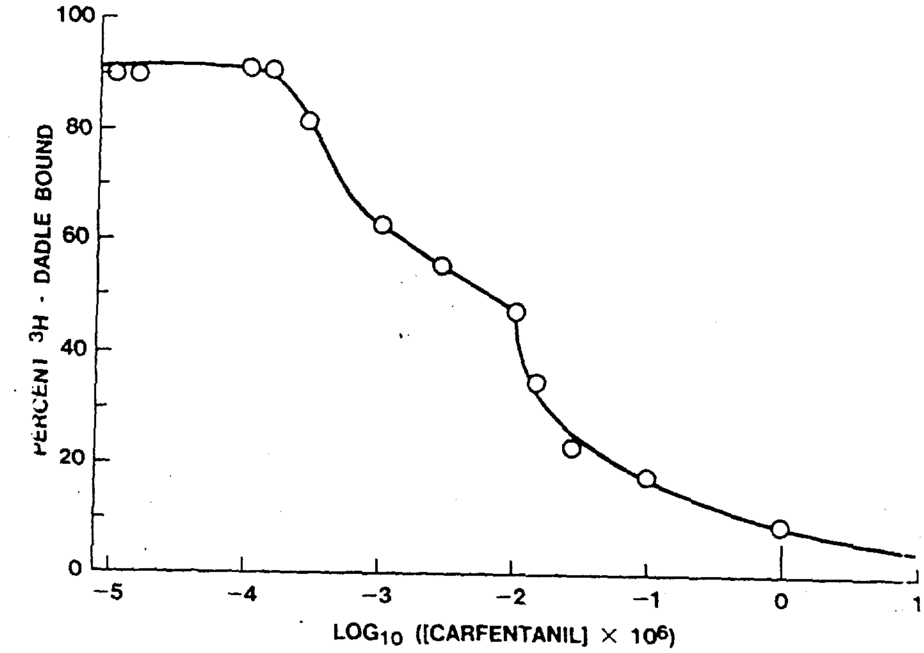

Figure 6. Displacement of ^{3}H-DADLE Binding by Carfentanil

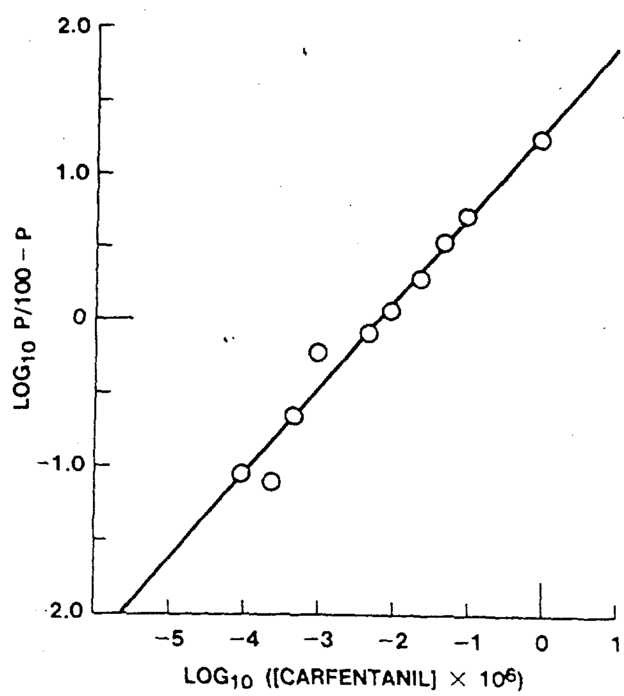

Figure 7. Hill Plot of ^{3}H-DADLE Displacement by Carfentanil

One of the more striking features of the data is the extreme potency with which carfentanil displaced both ^{3}H-DHM and ^{3}H-EKC. With an estimated K_I in the low picomolar range, carfentanil displays a 100-fold greater affinity than morphine for the MU and KAPPA receptors.[11] This potency ratio holds for both components of the biphasic inhibition curve. In contrast, carfentanil's potency in displacing ^{3}H-DADLE does not appear to differ appreciably from the values published for morphine.[12] Both compounds displace ^{3}H-DADLE in a biphasic manner with IC_{50}s in the low and mid-nanomolar range. In comparison with morphine, the greater affinity of carfentanil for the ^{3}H-DHM and ^{3}H-EKC labeled sites indicates carfentanil is 3 orders of magnitude more selective for the MU and KAPPA receptors than for the DELTA receptor. Note, however, that carfentanil's greater affinity for the MU and KAPPA receptors is accompanied by an apparent loss of selectivity between them. Whether one compares the IC_{50}, K'_D, or K_I values, carfentanil shows a selectivity ratio for the MU and KAPPA receptors in the range of 1-10. Morphine, on the other hand, can show a ratio of between 1 and 535, depending on whether the high or low affinity component of the displacement curve is used as the basis of comparison.[12,13] A selectivity index near unity for both high and low affinity components of the displacement curves for ^{3}H-DHM and ^{3}H-EKC, however, leads one to question whether carfentanil is actually equipotent at the MU and KAPPA receptors or whether the radioligand probes are simply labeling the same population of receptors.

Cross-labeling of the MU receptor by ^{3}H-EKC is known to occur and must be accounted for when attempting to infer the existence and pharmacologic uniqueness of a separate, topologically distinct KAPPA receptor. Several explanations for ^{3}H-EKC's labeling of the MU receptor must be considered. First is the possibility that EKC does not possess a sufficiently unique molecular structure to discriminate between the MU and KAPPA receptor binding sites. In this case, ^{3}H-EKC is a poor choice as an in vitro probe of KAPPA receptor pharmacology. Labeling of the MU receptor may also be anticipated from the claim of a single high affinity opioid binding site that binds all opiate-type compounds with near equal avidity, in which case little selectivity should be observed between the high affinity binding components of MU, KAPPA, and DELTA radioligands.[9-11] Although this argument is confirmed in the comparison between ^{3}H-DHM and ^{3}H-EKC binding, it is weakened by the high selectivity ratio (1666) obtained between carfentanil's displacement of ^{3}H-DHM and ^{3}H-DADLE. A third explanation for ^{3}H-EKC's apparent nonselectivity is found in a rather unique agonist-antagonist dualism in the pharmacologic profile of KAPPA compounds.

The observation that KAPPA compounds neither substitute for morphine nor precipitate withdrawal in the morphine-tolerant monkey led to the hypothesis of two distinct forms of the MU receptor: MU_1 and MU_2. The MU_1 receptor is thought to bind opioid compounds with high affinity and mediate the classic opiate effects of analgesia, catalepsy, prolactin release, and the turnover of acetylcholine. The MU_2 receptor binds opiates with lower affinity and mediates growth hormone release, respiratory depression, and metabolism of striatal dopamine.[15] A pharmacological dissociation between these MU receptor subtypes was reported in animals treated with naloxonazine, an opiate antagonist with high selectivity for binding to the MU_1 receptor. Rats treated with naloxonazine did not exhibit the expected analgesia after morphine but exhibited symptoms of MU_2 activation (e.g., respiratory

depression).[16] Other studies show that KAPPA compounds displace MU radioligands with high affinity in vitro, act as pure antagonists in the isolated rat vas deferens, block the morphine-induced increase in dopamine metabolism, and mitigate morphine's lethal effect in rats. These symptoms indicate a strong MU antagonism in the pharmacologic profile of putative KAPPA agonists.[15,17,18] The antagonist nature of KAPPA ligand binding is further evidenced by its relatively low sensitivity to inhibition by Na+, a standard feature for rating the agonist or antagonist properties of opiates in vitro.[19] From this perspective, a significant proportion of KAPPA ligand binding is expected to be in association with the MU receptor population -- partly as an agonist at the high affinity MU_1 site and partly as an antagonist to the low affinity MU_2 site.

The preference of KAPPA compounds to bind at the low affinity site is reflected in this study by the relative proportion of high and low affinity binding sites displaced by carfentanil. When ^{3}H-DHM was used as the receptor probe, the proportion of high-to-low affinity sites displaced by carfentanil was 50:50. When ^{3}H-EKC was used, the proportion shifted to 25:75 with no change in apparent IC_{50}s of either component. Although we can not determine from the data whether this low affinity binding site for ^{3}H-DHM and ^{3}H-EKC represents the MU_2 isoreceptor or the KAPPA receptor, if we assume the former to be correct then the implications of the data are twofold. First, the data show carfentanil to be approximately 10-20 times more potent than morphine at the MU_2 site. The 1000-fold increase in carfentanil's affinity for binding to the high affinity MU_1 receptor is thus partially offset by a 10-fold increase in affinity for the MU_2 receptor. Although the affinity of carfentenil for the MU_1 receptor has been highly correlated with decrease in the minimum effective dose to produce analgesia,[20] the increase in affinity for the MU_2 receptor may likewise explain the emergence of respiratory depression at higher dose levels necessary to achieve motor incapacitation and anesthesia. Second, the preferential binding of ^{3}H-EKC to the low affinity binding site may reflect the in vitro correlate of the antagonist properties of KAPPA compounds and predict their ability to antagonize the physiologic effects of MU_2 receptor activation. A preliminary investigation involving several compounds cited to be KAPPA agonists had revealed at least an ordinal correlation between their ability to displace ^{3}H-sufentanil in vitro and mitigate the lethal effects of morphine in mice.* Further studies are required to determine whether this antagonism of morphine's lethal effect, thought to be mediated by the MU_2 receptor, represents a specific pharmacologic antagonism at a common receptor or a physiologic antagonism through a separate KAPPA receptor-effector mechanism.

In summary, our results show that carfentanil possesses a very high affinity for at least two opioid binding sites. With the exception of a difference in the proportion of sites labeled, both sites were almost equally defined by the radiologands ^{3}H-DHM and ^{3}H-EKC. Carfentanil shows a much lower affinity for sites labeled with the DELTA receptor ligand, ^{3}H-DADLE, leading to the conclusion that carfentanil is highly selective for

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*Thompson, R.G., and Valdes, J.J., Research Directorate, U.S. Army Chemical Research, Development and Engineering Center, October 1987, unpublished data.

labeling the MU or KAPPA opioid receptor types. The failure of carfentanil to distinguish between distinct MU and KAPPA receptor sites was discussed within the context of EKC's pharmacologic role as a MU_2 antagonist. Based on these observations and considerations, we suggest that the MU_2 antagonist properties of various KAPPA compounds be further investigated, both in the context of their receptor binding profile at the MU_2 receptor and their physiologic efficacy for mitigating the respiratory depressant effects of various narcotics.

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